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An effective GC method for the determination of the fatty acid composition in silkworm pupae oil using a two-step methylation process

JUN WANG[1,2]*, WEIWEI WU[1], XUDONG WANG[1], MIN WANG[1] and FUAN WU[1,2]**

[1]School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, P. R. China and [2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, P. R. China

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Abstract: In the search for an accurate and effective method for the determination of the fatty acid composition in silkworm pupae oils, five methylation methods were evaluated for use in the gas chromatographic (GC) quantification of fatty acid methyl esters (FAMEs). These included two one-step acid-catalyzed (H_2SO_4 and BF_3) and two one-step base-catalyzed (KOH and $NaOCH_3$) esterification processes, as well as a two-step procedure catalyzed successively by KOH and H_2SO_4. These methods were comparatively adopted to quantify FAMEs in silkworm pupae oil using GC–MS and GC and then their precision, stability and average recovery rates were validated. The results indicated that compared with the four one-step methyl esterification methods, the two-step methylation effectively improved the synthesis yield of FAMEs, conserved the agents and eliminated the usage of potential harmful reagents. The proposed GC method was validated, exhibited good accuracy and precision, and was successfully applied to the quantification of FAMEs in several varieties of silkworm pupae oils. The short analytical run time leads to low costs and a fast chromatographic procedure. In summary, two-step pretreatment had superior performance, providing technical references for the determination and analysis of fatty acids in other oils.

Keywords: fatty acid; fatty acid methyl ester; gas chromatography; silkworm pupae oil; two-step methylation.

INTRODUCTION

Recently, much attention has been given to the silkworm (*Bombyx mori* L.) pupa as a human food source due to its nutritional value, especially in some Asian countries.[1] Silkworm pupae are reported to be 30 % oil and 50 % protein; thus, approximately 133–270 tons of oil is likely to be available annually in

Corresponding authors. E-mails: (*) wangjun@just.edu.cn; (**) fuword@163.com
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China as byproducts of the silk industry.[2] Silkworm pupae oil is also a highly nutritional and exploitable food resource that lowers cholesterol, improves memory and serves as an anti-oxidant by eliminating free radicals in the body.[3] The fatty acid composition of silkworm pupae oil varies according to its variety and origin, usually consisting of *α*-linolenic, oleic, linoleic, palmitic, palmitoleic and other fatty acids, with a total of more than 75 % unsaturated fatty acids (UFAs) and approximately 20 % saturated fatty acids (SFAs).[4] Among these fatty acids, *α*-linolenic acid, the precursor of n-3 polyunsaturated fatty acids (n-3 PUFAs), is an essential fatty acid that is found in high concentrations in certain plant oils, such as flaxseed, walnut, and canola oils. The beneficial effects of n-3 PUFAs on normal health and chronic disease, such as the regulation of lipid levels, cardiovascular disease, and immune function were elucidated.[5] Beneficially for humans, the proportion of saturated, monounsaturated and polyunsaturated fatty acids in silkworm pupae oil tends strongly to the optimum ratio of fatty acids recommended by current nutritionists for the human body. As the fatty acid composition is a major factor that influences the properties of silkworm pupae oil, the determination of fatty acids content is important.

There are several analytical methods that could be used for the identification and quantification of fatty acids in oils. These techniques include gas chromatography–mass spectrometry (GC–MS),[6] gas chromatography (GC),[7] high-performance liquid chromatography–mass spectrometry (HPLC–MS),[8] high-performance liquid chromatography (HPLC),[9] high-performance size exclusion chromatography (HPSEC),[10] nuclear magnetic resonance (NMR),[11] Raman spectroscopy,[12] Fourier transform-infrared spectroscopy (FTIR)[13] and near-infrared spectroscopy (NIR).[14] However, GC–MS and GC are the most widely used techniques for the determination of individual fatty acid profiles and contents, respectively, in vegetable and animal oils. Several studies showed that GC–MS,[15] and GC with flame ionization detection (GC–FID)[16] are also applicable for investigating the fatty acid profiles in silkworm pupae oil.

The critical process in GC analysis of fatty acids is the required methylation of the fatty acids to obtain fatty acid methyl esters (FAMEs). Many different methylation methods are described in the literature but the most commonly used are those catalyzed by an acid, base or boron trifluoride and methylation with diazomethane, each of which have advantages and disadvantages and differ in their applicable range.[17]

For the determination of fatty acids in silkworm pupae oil by GC, acid- or base-catalyzed and boron trifluoride methylation methods were reported. The advantage of acid-catalyzed and boron trifluoride methylation, in which H_2SO_4 and BF_3 are widely used, is that free fatty acids (FFAs) can be catalyzed for esterification.[18] However, higher temperatures, usually between 60 and 90 °C, and longer reaction times, which could affect the compositions of fatty acids,

easily lead to not only inaccurate results but also the occurrence of side reactions, loss of the unsaturated FAMEs and isomerization.[19] On the other hand, the advantages of base-catalyzed methylation are mild reaction conditions and fast reaction rate; however, there are two significant disadvantages. This method cannot catalyze the methyl esterification of FFAs, and the reaction must be conducted without water, which would otherwise often lead to saponification. In general, these methods are not necessarily convenient and must frequently be optimized for reaction conditions, including the catalyst and temperature. Thus, an accurate and effective method must be chosen for the methylation of fatty acids in silkworm pupae oil.

Silkworm pupae oils are lipid derivatives, so their compositions are very complex; therefore, an appropriate method is required to analyze their contents by converting both fatty acid salts and the acyl components in all lipid classes, such as triacylglycerols, phospholipids, sphingolipids, and waxes, to methyl esters using an effective esterification procedure. Recently, O'Fallon *et al.* presented a method to directly methylate fatty acids from muscle tissue, oils, and feedstuffs in aqueous solution that is based on a surprising concept, the addition of water to the fatty acid methyl ester synthesis reagents.[20] Usually, synthetic methods of FAMEs have rigorously avoided water as a matter of standard procedure. However, by adding water, the dynamics of sample preparation and methyl ester formation can be revisited, and the ideal outcome of the synthesis of FAMEs is enabled. Although the method described herein requires two steps, only one reaction tube is required. In summary, the developed protocol can simply obtain FAMEs from any sample. However, to the best of our knowledge, no report has been published concerning two-step methylation of fatty acids in silkworm pupae oil prior to GC analysis.

The aim of this work was to develop a validated method to determine the fatty acid composition in silkworm pupae oil by GC with two-step methylation. For this purpose, different varieties of silkworm pupae oils were extracted using a Soxhlet extractor, analyzed and determined by five methyl esterification methods, potassium hydroxide–methanol (KOH–MeOH), sodium methoxide–methanol ($NaOCH_3$–MeOH), sulfuric acid–methanol (H_2SO_4–MeOH), boron trifluoride–methanol (BF_3–MeOH), and a two-step methylation.

EXPERIMENTAL

Materials

Varieties of silkworm pupae including 5078, 220, 5082, HK3, and Qingsong×haoyue were supplied by the Sericultural Research Institute of the Chinese Academy of Agricultural Sciences.

The FAME standards, including methyl heptadecanoate (internal standard), methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate, were purchased from Sigma (St. Louis, MO, USA). Potassium hydroxide,

methanol, BF_3–MeOH (14 %), sodium methoxide, sulfuric acid, *n*-hexane, petroleum ether, and all other reagents and solvents were of analytical grade (Sinopharm Chemical Reagent, Shanghai, China). Water was purified using an Elga Purelab Option-Q purification system (Elga Labwater, High Wycombe, Buckingham, UK) and had a minimum resistivity of 18.0 MΩ cm.

Preparation of silkworm pupae oil samples

Silkworm pupae oil was extracted with petroleum ether using the Soxhlet method. Approximately 5 g of dried silkworm chrysalis and 80 mL of petroleum ether (60–90 °C) were used in each Soxhlet extraction, which was realized at 80 °C for 8 h. After the extraction procedure, the solvents were evaporated under vacuum, and the samples were subsequently stored at 4 °C.

Different methylation methods for FAME synthesis

*Two-step methylation***.** Lipids obtained after the extraction of silkworm pupae samples were converted to the corresponding FAMEs according to the literature.[20] In this procedure, 40 μL of silkworm pupa oil was placed into 10 mL centrifuge tubes to which 0.7 mL of potassium hydroxide (10 M) solution and 5.3 mL of methanol were added. The reaction was performed at 55 °C for 1.5 h with mixing for 5 s every 20 min. After cooling to room temperature, 0.58 mL of sulfuric acid (10 M) solution was added and the reaction was continued at 55 °C for 1.5 h with mixing for 5 s every 20 min. After cooling to room temperature, 3 mL of *n*-hexane was added and mixed for 5 min. Subsequently, the tubes were centrifuged for 5 min and the extracts were removed for GC analysis.

*BF_3–MeOH methylation***.** The methods and conditions of FAME preparation were realized according to the literature.[20] In this procedure, 40 μL of silkworm pupa oil was placed into 10 mL centrifuge tubes to which 2 mL of boron trifluoride in methanol solution (14 %) was added. The tubes were reacted at 55 °C for 1.5 h, with mixing for 5 s every 20 min. Then, 2 mL of saturated sodium bicarbonate solution and 3 mL of *n*-hexane were added, and the tubes were well mixed. The extracts were removed for GC analysis.

*$NaOCH_3$–MeOH methylation***.** The methods and conditions of FAME preparation were set up according to the literature.[20] Here, 40 μL of silkworm pupa oil was placed into 10 mL centrifuge tubes to which 2 mL of 0.5 M sodium methoxide solution was added. The tubes were reacted at 55 °C for 1.5 h with mixing for 5 s every 20 min. Then, 2 mL of saturated sodium bicarbonate solution and 3 mL of *n*-hexane were added, and the tubes were well mixed. The extracts were removed for GC analysis.

KOH–MeOH methylation. The methods and conditions of FAME preparation were set up according to the literature.[21] Here, 40 μL of silkworm pupa oil was placed into 10 mL centrifugal tubes to which 3 mL of KOH–MeOH solution (0.5 M) was added. The mixture was heated at 60 °C for 15 min. After cooling to room temperature, 3 mL of *n*-hexane and 2 mL of distilled water were added and mixed thoroughly. The extracts were removed for GC analysis.

H_2SO_4–MeOH methylation. The methods and conditions of FAME preparation were set up according to the literature.[21] In this procedure, 40 μL of silkworm pupa oil was placed into 10 mL centrifugal tubes to which 2 mL of H_2SO_4–MeOH solution (1 %) was added. The mixture was heated at 70 °C for 1 h. After cooling to room temperature, 3 mL of *n*-hexane and 2 mL of distilled water were added and mixed thoroughly. The extracts were removed for GC analysis.

Analysis of FAME products by GC–MS and GC

GC–MS analysis was carried out using an Agilent 6890 gas chromatograph with a 5973 MS detector equipped with 60 m×0.25 mm, i.d. 0.25 μm/MS DB-WAX capillary column (Agilent). The following temperature ramp was used: injector at 250 °C, oven initially at 200 °C, held for 1 min and heated to 230 °C (1.5 °C min^{-1}, then held for 10 min). The characterization and identification of FAMEs from silkworm pupae oil was completed in the SCAN mode with the *m*/*z* range varied from 35 to 450. The flow rate of the nitrogen as carrier gas was 1 mL min^{-1}; manual injection; the injection volume was 1 μL.

The fatty acid composition of the FAMEs from silkworm pupae oil was determined using an Agilent 6820 gas chromatograph equipped with a Supelco capillary column (hp-innowax, Agilent, 100 m×0.25 mm, i.d. 0.20 μm), a flame ionization detector and split injection port. The initial oven temperature was 200 °C, which was held for 1 min, subsequently increased to 230 °C at 1.5 °C min^{-1} and then held for 1 min. The injector was set at 250 °C, and the detector at 280 °C. Nitrogen was used as the carrier gas at a flow rate of 1 mL min^{-1}. The split ratio was 50:1, and the sample size was 1 μL.

Statistical analysis

Triplicate experiments were performed for each parameter investigated. The standard deviation of the measurements was calculated to check the reliability of the results. Statistical analysis was performed using the ANOVA method. Significant differences ($p < 0.05$) between the means were determined.

RESULTS AND DISCUSSION

Chromatogram analysis of FAME standards

With methyl C17:0 as the internal standard, GC–FID was used to analyze quantitatively the FAME content in the five types of samples of silkworm pupae oils. The chromatogram of the FAME sample prepared using the two-step methylation of the pupae oil from silkworm variety 5078 is shown in Fig. 1 as an example and the chromatograms of the FAME samples obtained by the single-step methylation procedures of the oil from the same silkworm variety are presented in Fig. S-1 of the Supplementary material to this paper. As could be seen,

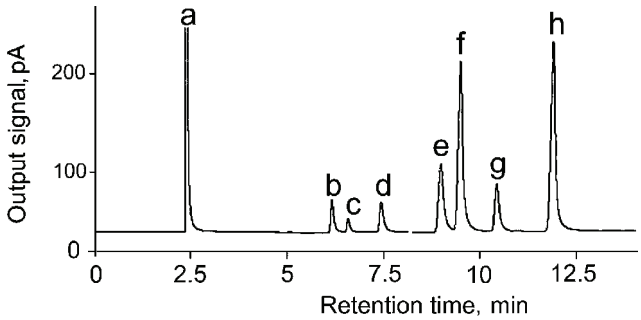

Fig. 1. GC chromatogram of FAMEs in pupae oil from silkworm variety 5078 prepared by the two-step methylation procedure. (a, *n*-hexane; b, methyl palmitate; c, methyl palmitoleate; d, methyl heptadecanoate; e, methyl stearate; f, methyl oleate; g, methyl linoleate; h, methyl linolenate).

all the main components of the samples were completely separated. The peaks were identified by comparison with the peaks of the chromatogram of the six FAME standards, which is shown in Fig. S-2 of the Supplementary material. All six FAME components, except for methyl heptadecanoate (internal standard), were also verified using MS and the obtained mass spectra are presented in Fig. S-3 (Supplementary material).

In the GC chromatograms, peaks at the same retention time represent the same compound, and the height and area of peaks directly reflect the content of each fatty acid methyl ester in the samples that indirectly responded to the effect of methyl esterification. The retention times of the FAME standards are listed in Table S-I of the Supplementary material. The results indicated that each fatty acid component in silkworm pupae oil was well separated and the analysis only took 12.5 min, avoiding thereby such problems as long analysis time and baseline drift. The short analytical run time leads to low costs and a rapid chromatographic procedure. Generally, the GC analysis method resulted in good separation, meeting both requirements of accuracy and precision.

Calibration curve for individual standards

The internal standard method was chosen as the quantification method to determine the response factor and concentration of each component present in the sample; methyl heptadecanoate was chosen as an internal standard. Response linearity was evaluated using gas chromatography, with a constant amount of the internal standard and varying amounts of the individual FAME standards. Standard FAME mixtures at different concentrations were prepared. In each standard mixture, 0.1 mg methyl heptadecanoate was added that was kept constant for all sample mixtures. Each sample preparation and subsequent injection and calculation for each concentration was performed in triplicate to verify the consistency, reliability and reproducibility of the GC method. The peak areas of the analyte and internal standard were calculated as the area ratio between the analyte and internal standard. The area ratio was plotted against the concentration ratio. The area ratios was calculated from the peak areas, which were automatically generated by the GC assistant software, and the concentration of each of FAMEs was calculated from the respective linear regression equations given in Table S-I of the Supplementary material to this paper. Table S-1 also includes the R^2 values and linear ranges for all six components. The results indicated that the six FAMEs (methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate) had good linearity ($R^2 > 0.9995$) in the ranges 1–10, 0.05–0.5, 0.25–2.5, 1–10, 0.5–2.5 and 1–10 mg mL^{-1}, respectively. Thus, the calibration curves for the individual FAME standards could be used to calculate and analyze the oil samples of silkworm pupae.

Comparison of the methylation methods for different silkworm pupae oil

The FAME contents of the five varieties of silkworm chrysalis oil that were obtained using the five methylation procedures are given in Table I. The results indicated significant differences among the five methylation methods. Employing the two-step methylation method always resulted in higher contents of FAMEs than those using the KOH–MeOH, $NaOCH_3$–MeOH, H_2SO_4–MeOH, or BF_3––MeOH methylation procedures ($p > 0.05$).

TABLE I. FAME contents (mg mL^{-1}) in pupae oil from five varieties of silkworm obtained using the five methylation methods; a–e: the mean values in the same row for FAME contents in silkworm pupae oil using varying reaction condition are significantly different ($p < 0.05$)

Variety	Method	Component					
		Methyl palmitate	Methyl palmitoleate	Methyl stearate	Methyl oleate	Methyl linoleate	Methyl linolenate
5078	KOH	0.822 ±0.026^{c}	0.060 ±0.001^{c}	0.234 ±0.025^{c}	0.815 ±0.116^{e}	0.520 ±0.020^{d}	1.453 ±0.150^{c}
	H_2SO_4	1.221 ±0.034^{b}	0.099 ±0.002^{b}	0.324 ±0.003^{b}	1.827 ±0.031^{b}	0.651 ±0.006^{b}	2.288 ±0.052^{b}
	BF_3	0.626 ±0.055^{de}	0.054 ±0.005^{cd}	0.189 ±0.011^{de}	0.918 ±0.020^{c}	0.506 ±0.015^{ed}	1.374 ±0.046^{cd}
	$NaOCH_3$	0.517 ±0.022^{e}	0.039 ±0.003^{e}	0.185 ±0.006^{e}	0.835 ±0.039^{cd}	0.522 ±0.007^{cde}	0.976 ±0.047^{e}
	Two-step	1.425 ±0.083^{a}	0.128 ±0.006^{a}	0.370 ±0.018^{a}	2.227 ±0.053^{a}	0.741 ±0.033^{ab}	2.756 ±0.084^{a}
220	KOH	0.976 ±0.113^{c}	0.067 ±0.006^{d}	0.200 ±0.006^{c}	1.365 ±0.068^{c}	0.536 ±0.009^{c}	1.439 ±0.072^{c}
	H_2SO_4	1.479 ±0.005^{b}	0.113 ±0.003^{b}	0.270 ±0.003^{c}	1.992 ±0.007^{b}	0.673 ±0.001^{b}	2.232 ±0.008^{b}
	BF_3	0.739 ±0.052^{e}	0.059 ±0.001^{d}	0.167 ±0.006^{b}	0.982 ±0.054^{d}	0.518 ±0.015^{c}	1.349 ±0.092^{d}
	$NaOCH_3$	0.765 ±0.069^{de}	0.099 ±0.007^{c}	0.228 ±0.008^{c}	1.099 ±0.098^{d}	0.487 ±0.016^{c}	1.164 ±0.851^{d}
	Two-step	2.155 ±0.080^{a}	0.193 ±0.012^{a}	0.344 ±0.010^{a}	2.978 ±0.091^{a}	0.879 ±0.018^{a}	3.314 ±0.086^{a}
5082	KOH	0.660 ±0.041^{d}	0.044 ±0.004^{e}	0.211 ±0.002^{cd}	1.101 ±0.064^{d}	0.495 ±0.010^{d}	1.219 ±0.010^{d}
	H_2SO_4	1.392 ±0.061^{ab}	0.093 ±0.004^{b}	0.367 ±0.014^{b}	2.206 ±0.047^{ab}	0.732 ±0.011^{ab}	2.662 ±0.072^{b}
	BF_3	0.505 ±0.120^{de}	0.048 ±0.003^{de}	0.172 ±0.003^{de}	0.796 ±0.022^{e}	0.479 ±0.010^{de}	1.118 ±0.044^{de}
	$NaOCH_3$	0.933 ±0.134^{c}	0.067 ±0.013^{c}	0.277 ±0.019^{c}	1.564 ±0.149^{c}	0.583 ±0.025^{c}	1.780 ±0.158^{c}
	Two-step	1.581 ±0.128^{a}	0.114 ±0.002^{a}	0.402 ±0.004^{a}	2.461 ±0.010^{a}	0.782 ±0.008^{a}	3.075 ±0.030^{a}

TABLE I. Continued

Variety	Method	Component					
		Methyl palmitate	Methyl palmitoleate	Methyl stearate	Methyl oleate	Methyl linoleate	Methyl linolenate
Jingsong ×haoyue	KOH	0.727 ±0.055^{cd}	0.056 ±0.004^{c}	0.187 ±0.009^{c}	0.959 ±0.059^{c}	0.502 ±0.016^{c}	1.553 ±0.053^{c}
	H_2SO_4	1.138 ±0.008^{ab}	0.075 ±0.002^{b}	0.257 ±0.001^{b}	1.400 ±0.012^{b}	0.630 ±0.006^{b}	2.496 ±0.019^{b}
	BF_3	0.431 ±0.069^{e}	–^{e}	0.141 ±0.005^{e}	0.584 ±0.031^{e}	0.440 ±0.023^{de}	1.038 ±0.078^{e}
	$NaOCH_3$	0.767 ±0.111^{c}	0.050 ±0.008^{cd}	0.185 ±0.005^{cd}	0.809 ±0.058^{cd}	0.470 ±0.039^{d}	1.320 ±0.103^{d}
	Two-step	1.385 ±0.023^{a}	0.105 ±0.001^{a}	0.283 ±0.015^{a}	1.742 ±0.029^{a}	0.701 ±0.009^{a}	3.157 ±0.037^{a}
HK3	KOH	0.531 ±0.026^{d}	0.040 ±0.006^{d}	0.157 ±0.001^{d}	0.832 ±0.005^{d}	0.445 ±0.020^{d}	0.913 ±0.001^{d}
	H_2SO_4	0.958 ±0.059^{b}	0.066 ±0.007^{bc}	0.213 ±0.006^{b}	1.325 ±0.090^{b}	0.562 ±0.017^{b}	1.412 ±0.081^{b}
	BF_3	0.428 ±0.014^{de}	–^{e}	0.135 ±0.003^{e}	0.605 ±0.025^{e}	0.423 ±0.002^{de}	0.674 ±0.023^{e}
	$NaOCH_3$	0.871 ±0.140^{c}	0.071 ±0.001^{b}	0.184 ±0.038^{c}	1.259 ±0.345^{bc}	0.505 ±0.041^{c}	1.198 ±0.253^{c}
	Two-step	1.101 ±0.124^{a}	0.098 ±0.007^{a}	0.248 ±0.018^{a}	1.723 ±0.166^{a}	0.651 ±0.028^{a}	1.937 ±0.078^{a}

This result is closely related to the nature of silkworm pupae oil. There is a certain amount of free fatty acid in silkworm pupae oils, more than 2 mg KOH g^{-1}.[21] In general, FFAs always produce negative effects because their presence causes soap formation that consumes catalyst and reduces the catalytic effectiveness,[22] which leads to an increased solubility of FAMEs in the glycerol layer. Overall, the effects of KOH or $NaOCH_3$ catalysts were not suited for methylation of the highly acidic oils. Moreover, BF_3 methylation has often been applied to test neutral components, but its results were affected by reaction time.[23] However, BF_3 is not suitable for the transesterification of triglycerides due to its potential for producing many artifacts.[24,25] Furthermore, in the presence of sulfuric acid as a catalyst, the method is suitable for the methylation of FFAs.[21] However, the reactions catalyzed by sulfuric acid are generally time-consuming, require high temperatures and did not result in complete conversion. In the two-step methylation, the first step was to hydrolyze fatty acid esters to FFAs under alkaline conditions, and the second step was esterification of the formed FFAs with methanol. Although the method consisted of two steps, the whole process was completed in one container. This methylation method was different from the others because some water was added to the reaction; water is known to facilitate the reaction.[20] Therefore, for FAME synthesis from silkworm pupae oil, the two-step methylation was superior among the five tested methods.

Method validation and precision

The precision of the five methylation methods was evaluated in terms of repeatability, which was expressed as relative standard deviation (*RSD*). The precision in terms of repeatability was obtained at different levels by the analysis of the FAMEs in pupae oil from silkworm variety 5078. The ANOVA of the chrysalis oil FAME was calculated using PROCGLM (SAS Inst. Inc., Cary, NC, USA).[26] The precision test results of FAME content of pupae oil from silkworm variety 5078 are given in Table S-II of the Supplementary material. The percent *RSD* of the FAME content with the two-step synthetic method ranged from 2.39 to 4.97 %, except for methyl palmitate (5.82 %). The FAME precisions with the other methods were between 1 and 11 %, while less than 3 % *RSD* was observed with the acid catalyst H_2SO_4. In addition, the methyl linoleate precisions of the five methylation methods (0.98–4.44 %) were higher than those for the other FAMEs. The *p*-values for the five methylation methods were all less than 0.05, indicating that the difference in the data was significant and that the data were valid.

Stability

A stability test was conducted on the five methylation methods of the pupae oil from silkworm variety 5078 after a week, and the corresponding stability analysis is given in Table S-III of the Supplementary material. Compared with the data in Table I, the content of all FAMEs produced by five methylation methods in Table S-III were slightly increased after one week. Using the two-step method, the contents of C16:0 and C18:3 were increased from 1.425±0.058 mg mL^{-1} to 1.909±0.009 mg mL^{-1} and from 2.756±0.084 mg mL^{-1} to 3.641± ±0.010 mg mL^{-1}, respectively. Thus, there is a possible correlation between the increase in FAMEs content and the precision of five methyl esterification methods. However, using the H_2SO_4–MeOH methylation, the fatty acid content remained essentially unchanged after a week. For example, the content of C16:0 was just decreased from 1.392±0.061 to 1.302±0.005 mg mL^{-1} ($p < 0.05$). This is because the fatty acids easily deteriorate after a few days of unfavorable conditions due to their poor stability.[27] Therefore, it was inferred that the samples of silkworm pupae oil should be determined as soon as possible.

Average recovery

After methyl esterification by the five methylation methods, the FAMEs standards were added to the samples to achieve 80, 100 and 120 % of the corresponding amount in the samples. GC was used to detect the total amount of the FAMEs, and the recovery rate was calculated. A summary of the average recovery rates of FAMEs in pupae oil obtained from silkworm variety 5078 using the five methylation methods is presented in Table S-IV of the Supplementary mater-

ial. The average recovery rates of FAMEs ranged from 114.30 to 120.66 % for methyl palmitate, from 80.62 to 116.01 % for methyl palmitoleate, from 100.91 to 119.96 % for methyl stearate, from 89.36 to 108.77 % for methyl oleate, from 114.40 to 123.31 % for methyl linoleate and from 76.13 to 85.02 % for methyl linolenate. Except for the recovery rate of methyl linoleate using the H_2SO_4––MeOH methylation (123.31 %), the average recovery rate of methyl linolenate using the two-step method was the highest (85.02 %), and the average recovery rates of other FAMEs were tolerable (75–120 %). There were significant differences in the *RSD*. The *RSD* of the two-step method was lower than those of the other methods, which indicated that the reproducibility using the two-step method was more favorable.

Therefore, the results above indicate that FAME formation and GC analysis using two-step methylation of pupae oil from silkworm variety 5078 had high average recovery rates, with acceptable values of the *RSD* percentage. Importantly, this method is more effective and has a better reproducibility than the other methods as water could be used and was not antagonistic to the methylation. Lengthy preparation times are not required for lyophilization a sample (usually takes days) or perform prior organic solvent extractions and no evaporations (usually hours), which are required to eliminate water in the other fatty acid methods.[20] In addition, the two-step methylation not only improves the FAMEs yield, but it also eliminates the potential harmfulness of solvents.

Determination of oil content and fatty acid composition in different silkworm pupae

The Soxhlet extracted weights of the oils from different silkworm pupae samples are given in Table II. Among the five varieties of silkworm pupae, the oil content of silkworm variety HK3 was the greatest (56.4 %); the least was that of silkworm variety 220 (27.4 %), while the others were between 30 and 40 %. Therefore, the result corresponds with those previously reported that the oil contents of different varieties of silkworm pupae were significantly different.[2]

TABLE II. Pupae oil weights of five varieties of silkworm

Variety	Oil weight, g/5 g of dried silkworm chrysalis[a]	Oil content, %
5078	1.58	31.6
220	1.37	27.4
5082	1.71	34.2
HK3	2.82	56.4
Jingsong × haoyue	1.99	39.8

[a]80 mL petroleum ether, 80 °C for 8 h

In addition, as shown in Table I, the fatty acid contents in the oils from different varieties of silkworm pupae determined by the same methylation procedures were significantly different, suggesting that fatty acid compositions were

different in different varieties of silkworm pupae. As a whole, the fatty acid components in silkworm pupae oil were palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic whereby the content of linolenic acid was the highest. The contents of linolenic acid determined using the two-step method were clearly higher than those determined using the other methylation methods. Among the oils from the five varieties of silkworm pupae, the content of linolenic acid in the oil from silkworm variety 220 was the highest (3.314±0.086 mg mL^{-1}).

The obtained results indicate that silkworm pupae oil is a good source of linolenic acid. As many human diets are deficient in n-3 PUFAs, enrichment of food products is an alternative to increasing the intake of these fatty acids.[2] Thus the procurement of linolenic acid concentrate from silkworm pupae is important for pharmaceutical and dietary purposes.

CONCLUSIONS

A quantitative method for preparing FAMEs for assessing the fatty acid content of silkworm pupae oils using GC with two-step methylation was developed. Compared with the other four methyl esterification methods (KOH, $NaOCH_3$, H_2SO_4, BF_3–MeOH methylation), the two-step methylation not only simplifies the experimental procedure of FAME synthesis and saves chemicals, but also eliminates the employment of potential harmful reagents. The proposed GC–FID method was validated and successfully applied to the quantification of FAMEs in the oils from various varieties of silkworm pupae. Thus, the developed methodology could be used for the convenient and effective determination of FAMEs in silkworm pupae oil and other samples.

SUPPLEMENTARY MATERIAL

GC chromatograms of FAMEs, Figs. S-1–S-3, details on statistics, Tables S-I and S-II, as well as FAME content in silkworm pupae oil, Table S-III, are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ИЗВОД

УСПЕШНИ ГАСНО–ХРОМАТОГРАФСКИ МЕТОД ЗА ОДРЕЂИВАЊЕ САДРЖАЈА МАСНИХ КИСЕЛИНА У УЉУ ЛУТКЕ СВИЛЕНЕ БУБЕ ПРИМЕНОМ ДВОСТЕПЕНОГ МЕТИЛОВАЊА

JUN WANG[1,2], WEIWEI WU[1], XUDONG WANG[1], MIN WANG[1] и FUAN WU[1,2]

[1]*School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, P. R. China и*
[2]*Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, P. R. China*

У потрази за успешним методом одређивања масних киселина гасном хроматографијом (GC) у уљу лутке свилене бубе, испитано је пет метода метиловања за добијање метил-естара масних киселина (FAME), укључујући једностепену естерификацију катализовану киселином (H_2SO_4 или BF_3) или базом (KOH или $NaOCH_3$), као и двостепену

катализовану прво са KOH, па H_2SO_4. Ови поступци метиловања су упоређивани у методама GC–MS и GC, а затим валидирани кроз прецизност, стабилност и повраћај. Резултати су показали да се поступком двостепеног метиловања добија већи принос FAME, постиже се већа стабилност и избегава се употреба потенцијално штетних реагенаса. Предложени GC метод је испољио задовољавајућу тачност и прецизност и успешно је примењен за одређивање FAME у неколико варијетета уља свилене бубе. Кратко време аналитичког корака смањује трошкове и убрзава хроматографски поступак. У закључку, двостепено метиловање је имало боље перформансе у односу на једностепено, отварајући могућност примене ове методе за анализу масних киселина и из других уља.

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